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From the vagus nerve to the ventral tegmental area: a pathway for post-ingestive food reinforcement in the development of obesity

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Resumo

Atualmente a obesidade é considerada uma epidemia mundial que atinge os países desenvolvidos, trazendo inúmeros problemas de saúde, com consequências na qualidade de vida e esperança média de vida. O consumo excessivo e compulsivo de comida são comportamentos que se observam na obesidade. Existem sinais periféricos e neuronais que regulam o consumo alimentar. São exemplos de sinais periféricos a leptina, insulina e grelina que alteram o consumo alimentar por acção directa em centros neuronais que controlam o comportamento alimentar. O hipotálamo é a estrutura responsável pela regulação destes mecanismos homeostáticos de controlo alimentar, contudo sabemos hoje que sinais periféricos humorais (de natureza hormonal ou através do sistema nervoso autónomo) tem também importância noutras zonas cerebrais que são responsáveis pela sensação de prazer. A dopamina é o principal neurotransmissor responsável por modular mecanismos de recompensa alimentar através do sistema mesolímbico. A dopamina é sintetizada nos neurónios dopaminérgicos que se encontram em duas regiões adjacentes, a área tegmental ventral (ATV) e a substanica nigra pars compacta (SNc) e que por sua vez projectam os seus axónios para diversas regiões, principalmente para o estriado, constituindo os vários sistemas dopaminérgicos, responsáveis pela regulação da motivação, funções executivas, controlo motor, reforço e recompensa. A dopamina após libertação no terminal sináptico actua maioritariamente em dois tipos de receptores, receptores D1 e receptores D2.
Os mecanismos dopaminérgicos envolvidos na regulação do consumo alimentar não são ainda totalmente compreendidos. Contudo, estudos em humanos evidenciam que em obesos existe uma correlação negativa entre a concentração receptores de dopamina, especificamente D2 no estriado e o índice de massa corporal (IMC), sugerindo uma alteração ao nível da sinalização de dopamina associada à obesidade.

Sabe-se que comportamento alimentar rege-se por duas componentes, a componente orosensorial, relativa a palatibilidade, cheiro e aspecto dos alimentos, e por outro lado uma componente mais relacionada com o conteúdo nutricional e/ou calórico do alimento ingerido, através de processos que ocorrem após deglutição dos alimentos. Estes mecanismos são designados mecanismos pós-ingestivos. A evidência de que o consumo de alimentos de elevada palatibilidade leva a alterações nos circuitos associados a recompensa alimentar foi vastamente estudada. Contudo não existe até à data o conhecimento de uma relação causal entre o aumento da actividade destes neurónios e mecanismos pós-ingestivos. Mais ainda a importância destes mecanismos na actividade dopaminérgica e a sua relevância em patologias como a obesidade são até à data desconhecidos. Estes são assim os principais objetivos desta tese. Pretendeu-se estudar a fisiologia dos mecanismos pós-ingestivos e a activação de neurónio dopaminérgicos na ATV. Desta forma foi-se avaliar a actividade de neurónios dopaminérgicos da ATV enquanto diferentes estímulos isocalóricos eram administrados directamente ao estômago de ratinhos. Foram testadas 4 diferentes soluções: 20% sucrose como fonte de carbohidratos; 9% óleo de milho como fonte de gordura; 20% ácido glutâmico como fonte de proteína, e ainda 0.2% de sucralose como solução doce não calórica. A utilização de técnicas genéticas associadas a técnicas inovadoras de microscopia permitiram assim desenvolver um protocolo comportamental com o animal acordado e a movimentar-se livremente. Observamos que apesar dos neurónios da ATV aumentarem a sua actividade após a ingestão de um estímulo gástrico, independente do conteúdo nutricional apenas da administração intragástrica de 20% sucrose aumentou significativamente a actividade dos neurónios dopaminérgicos na ATV. Estes resultados sugerem que carbohidratos como a sacarose activam especificamente áreas de recompensa alimentar como a ATV que não são observáveis com outros macronutrientes, nomeadamente gordura e proteína.
Adicionalmente, pretendia-se estudar estes mecanismos pós-ingestivos e regulação dopaminérgica da ATV após indução de obesidade. Para iniciarmos este segundo objectivo foi necessário fazer um estudo prévio para determinar a melhor dieta que garantisse que os animais 1) aumentavam de peso; 2) que este aumento de peso ocorreria num espaço temporal rápido para que fosse possível fazer a medição de actividade dos neurónios dopaminérgicos na ATV sem que ocorresse perda de actividade ao longo do tempo. Foram testadas duas dietas sólidas e uma dieta líquida. Das dietas sólidas testámos uma dieta cujo 60% do teor calórico era de proveniência da gordura. Uma segunda dieta sólida em que cujo 70%

do teor calórico provinha de carbohidratos. Na dieta líquida a água foi substituída por uma solução de 35% de sacarose. Durante 12 semanas mediu-se peso consumo de comida e de líquidos. Dos resultados obtidos foi possível obter alterações significativas de peso utilizando duas dietas: uma dieta rica em gordura (60%) e dieta líquida com 35% de sacarose. Em ambos os casos os animais diminuíram significativamente o consumo alimentar. No caso da dieta líquida de 35% sacarose ocorreu o aumento do consumo desta solução ao longo do protocolo de obesidade. Comparando todas as dietas testadas observámos que a dieta que temporalmente aumentou significativamente o peso foi a dieta sólida com 60% de gordura. Destas duas dietas provavelmente iremos utilizar a dieta líquida pois com uma dieta líquida poderemos perfundir directamente no estômago e perceber o impacto da estimulação pós-ingestiva na regulação dos neurónios dopaminérgicos da ATV ao longo do ganho de peso.
Num segundo objectivo e de forma a perceber a importância dos mecanismos pós ingestivos no ganho de peso foram utilizados ratinhos mutantes (trpm5) cuja via de transdução de sinal de palatibilidade para o doce não se encontra presente. Nestes animais são os mecanismos pós-ingestivos que regulam o comportamento alimentar. Neste grupo de animais após dieta líquida de 35% de sacarose observou-se um aumento de peso similar ao aumento de peso observado em ratinhos não mutantes indicando que são maioritariamente os mecanismos pós-ingestivos que parecem regular o aumento de peso induzido pela dieta líquida de 35% sucrose.
O desenvolvimento desta dissertação permitiu assim concluir que mecanismos pós-ingestivos activam neurónios dopaminérgicos na ATV de uma forma especifica, nomeadamente para carbohidratos.
Ratinhos submetidos a diferentes dietas tem alterações diferenciais no ganho de peso, e que dietas ricas em gordura e/ou dieta líquida rica em carbohidratos são susceptíveis a ganho de peso. Finalmente em ratinhos mutantes onde a componente orosensorial para o doce são desactivadas tem um perfil de ganho de peso semelhante a animais não mutantes sugerindo que são maioritariamente os mecanismos não sensoriais que regulam o peso.

Palavras-chave: Obesidade, dopamina, recompensa alimentar, estímulos pós-ingestivos

Abstract

Obesity is a global epidemic affecting developed countries, bringing consequences to health and quality of life. Excessive and compulsive food intake are characteristic of obesity, however the mechanisms involved in regulating food intake remains unclear. Food behavior is governed by two components, orosensorial (palatability) and another related to nutritional and/or caloric content, through processes that occur after food has been swallowed, namely post-ingestive mechanisms. There is no knowledge of a relationship between dopamine synthesis/activation and post-ingestive mechanisms, and the impact of those mechanisms in obesity.

The aim of this project was to understand how dopaminergic neurons in the Ventral Tegmental Area (VTA) respond to different post-ingestive stimuli. More specifically we aimed to understand if VTA dopamine neurons respond differently according to the intragastric (IG) reinforcer administered. 3 different intragastric solutions were administered to the stomach and simultaneously VTA dopaminergic neurons activity was recorded before and after the injection. The reinforcers infused where isocaloric and differ in the nutrient infused: IG fat (9% corn oil) reinforcer, IG carbohydrate (20% sucrose) reinforcer and IG protein (20% glutamic acid) reinforcer. Our results showed that VTA dopaminergic neurons were specifically activated when sucrose was infused into the stomach indicating that post-ingestive dependent dopamine responses in the VTA are carbohydrate specific.

In a second aim we propose to evaluate and understand how post-ingestive mechanism modulate weight gain and a causal relation between those mechanism and VTA dopaminergic neurons. Before starting this second aim several diets were tested in order to induce weight gain. High-Fat diet and the liquid diet of 35% sucrose significantly increased animal weight. In a third aim mutant mice were used, where the sweet taste perception was abolished. In these animals weight gain was similar to the control littermates indicating that the orosensorial component of food intake is not necessary to induce obesity.

Keywords: Obesity, dopamine, food reward, post-ingestive mechanisms

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List of abbreviations

AADC - aromatic L-amino acid decarboxylase 13
ARH - Arcuate nucleus of hypothalamus 12
B6 - C57bl/6J mice 17
BMI - body mass index 10
CART - cocaine- and amphetamine-regulated transcript 12
CNMF-e - constrained nonnegative matrix factorization for endoscopes 2
DA - dopamine 13
DAT - Dopamine transporter 14
Dat-cre - B6.SJL-Slc6a3$^{tm1.1(cre)Bkmn}$/J 17
DIO - diet induced obesity 22
GRIN - gradient-index 19
HC - High-Carbohydrate 18
HF - High-Fat 18
IOTF - international obesity task force 11
L-DOPA - L-l-3,4-dihydroxyphenylalanine 13
LED - light-emitting diode 20
LH - lateral hypothalamus 12
MRI - magnetic resonance imaging 16
NAc - nucleus accumbens 13
NPY - neuropeptide Y 12
PET - positron emission tomography 16
POMC - pro-opiomelanocortin 12
PVN - paraventricular nucleus of hypothalamus 12
SNc - substancia nigra pars compacta 14
Tprm5 - 129-Trpm5^{tm1Csz}/J 17
VMH - ventro-medial hypothalamus 12
VTA - Ventral tegmental area 5

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1. Introduction

1.1 Obesity: A global epidemic

According to the World Health Organization (WHO), obesity is classified as a body mass index (BMI) higher than 30 kg / m^2 and is associated with an increased risk of cardiovascular diseases, diabetes, cancer among others, and reveals negative consequences for quality and average life expectancy (Seidell, 2002; Tsigos et al., 2008). Over the past 30 years, the increased consumption of palatable and energy dense food exacerbated these numbers and the prevalence of obesity has increased dramatically in most developed countries, leading the WHO and the International Obesity Task Force (IOTF) to declare a global obesity epidemic (Seidell, 2002; Taubes, 1998). Thus, Obesity has become one of the greatest threats to public health, resulting in high annual costs to the healthcare systems, including personal healthcare, medical services and medications (Allison et al., 1999). Portugal is not an exception and the numbers are also alarming, and more than 20% of the population is obese (Oliveira et al., 2018).

Guidelines for the management of obesity were created introducing strategies to improve diet nutritional content and promotion of physical activity (Tsigos et al., 2008). But clearly this type of interventions fails in long-term sustaining weight loss. One of the most effective treatment for morbid obesity regarding long-term weight loss, life quality and decrease of mortality, is bariatric surgery, namely the gastric bypass surgery. In this surgical approach the stomach is resected into a small stomach pouch and re-routed to the lowest part of the duodenum (Shin et al., 2011; Tsigos et al., 2008). The initial malabsorption mainly of carbohydrates in the upper part of the intestine and the total alteration of feeding patterns (Hammer, 2012), posed this intervention in the main focus of research and opened a new research area more related to food post-absorptive properties (Shin et al., 2011).

Besides the awareness of the obesity numbers, the pathophysiology behind overeating is still unknown. What causes overeating patterns is still unknown and a multidisciplinary approach is probably the way to roadmap this disease, converging information from metabolic, hormonal, humoral and neuronal pathways that directly or indirectly control food intake. Understanding these physiological mechanisms can probably give new insights into the aetiology of this pathology and mark new effective and non-invasive therapies to treat obesity.

1.2 Homeostatic regulation of food intake

Regulation of appetite and body weight involves a complex interaction between multiple neural systems, peripheral energy stores and external cues that influence food availability and palatability. In order to establish energy balance, homeostatic neuronal circuits in the central nervous system integrates two major peripheral factors that are triggered upon food ingestion. These include hormonal signalling and neuronal mechanisms.

Hormonal signalling: Insulin, Ghrelin and Leptin

Insulin is a hormone synthetized and released by the pancreas, acting in response to glucose plasma levels (Stanley et al., 2005). The receptors of this hormone can be found in liver, skeletal muscle and adipose tissue promoting glucose uptake. Insulin receptors can also be found in several brain nuclei although their neuronal function, besides decreasing food intake (through hypothalamic action), is still

unknown. Insulin is an anorexigenic hormone, meaning that when released it decreases food intake. This effect is mediated through the hypothalamus (Won et al., 2009).

When the stomach is empty ghrelin hormone is discharged, in order to increase food intake, re-establishing energetic balance. Therefore, this hormone acts to induce food intake (orexigenic) and rapidly decrease with the ingestion of food (Wynne et al., 2005).

The adipose tissue is an active endocrine organ, releasing several hormones. Leptin has been the most studied since signals of this hormone control food intake (anorexigenic) (Won et al., 2009). Leptin is highly correlated with adipose tissue mass regulating mostly long-term food intake, the more stored fat, the more leptin is secreted. In case of sustained food deprivation, the action of this hormone is suppressed, being released again after the weight and energy balance is restored (Stanley et al., 2005). In most obese individuals, despite increased leptin plasma levels the efficacy of the anorexic effect of leptin is decreased, leading to a state of leptin resistance (Farr et al., 2015). There are several molecular mechanisms of leptin resistance described in obesity, such as diminished number of leptin receptor, alterations in the leptin transport through the blood brain barrier and/or defects in intracellular signalling associated with leptin receptor (Schwartz et al., 2000; Zhou and Rui, 2013; Gruzdeva et al., 2019).

Collectively, energy homeostasis involves the central integration of peripheral hormonal signals that include short-term regulators where its effects occur during meal ingestion or fasting periods, such as insulin and ghrelin, respectively and long-term afferent signals from the adipocytes in case of leptin. Further, all those hormones regulate food intake through a highly regulated brain area, the hypothalamus, discussed above (McMinn et al., 2000). In addition, insulin, and leptin receptors have been described in other brain nuclei such as hippocampus, where these hormones seem to play an important role in cognitive function, including learning and memory (Grillo et al., 2015; Van Doorn et al., 2017; Hsu et al., 2018) and in VTA where stimulatory and inhibitory actions on dopamine neurons have been reported (Murakami et al., 2018; Liu and Borgland, 2019).

Neuronal mechanisms: The Hypothalamus

Hypothalamus is a brain area that contains multiple neuronal circuits highly connected to each other, and implicated in a variety of functions, mostly associated to maintaining body homeostasis. One of the most important functions is regulating food intake and energy homeostasis.

One particular important region, where leptin, insulin and ghrelin receptors are expressed, is the arcuate nucleus of the hypothalamus (ARH), given its role in regulating feeding (Wynne et al., 2005). In the ARH there are two populations of neurons. One population expresses the peptide neurotransmitter pro-opiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART), that when activated will inhibit food intake and increase metabolic rate. Another population expresses neuropeptide Y (NPY) and agouti-related peptide (AgRP), these neurons will stimulate eating. Insulin and leptin suppress NPY/AgRP neurons while stimulating POMC neurons, thereby decreasing food intake (Cowley et al., 2001). Ghrelin also binds to ARH receptors, but functions in the opposite manner leading to food consumption (Lutter and Nestler, 2009).

The ARH projects to secondary neurons within the hypothalamus, such as the paraventricular nucleus (PVN), dorsomedial hypothalamus, lateral hypothalamus (LH), and ventral medial hypothalamus (VMH) (Wynne et al., 2005). In the LH, several peptides are synthesised and released under the influence of NPY/POMC neurons projections from the ARH (Näslund and Hellström, 2007). These

neuropeptides modulate food intake via projections to various brain areas secondary to the homeostatic control, associated with arousal, reward and motivation (brainstem, nucleus accumbens, prefrontal cortex, ventral tegmental area (Cason et al., 2010)).

In conclusion, the hypothalamus major function is regulating homeostatic mechanisms, such as control of food intake and energy expenditure. Figure1.1. illustrates the interaction between the hormonal signalling and neuronal mechanisms in food intake regulation. In addition, it has also been described that this brain area connects to higher order neurons in several other brain regions. Albeit the homeostatic balance strongly influences and controls eating behaviour, it can also be influenced by other systems, such as non-homeostatic systems (Liu and Kanoski, 2018; Rossi and Stuber, 2018; Godfrey and Borgland, 2019). The 'hedonic control of eating' is often referred to the processes that controls eating without homeostatic need.

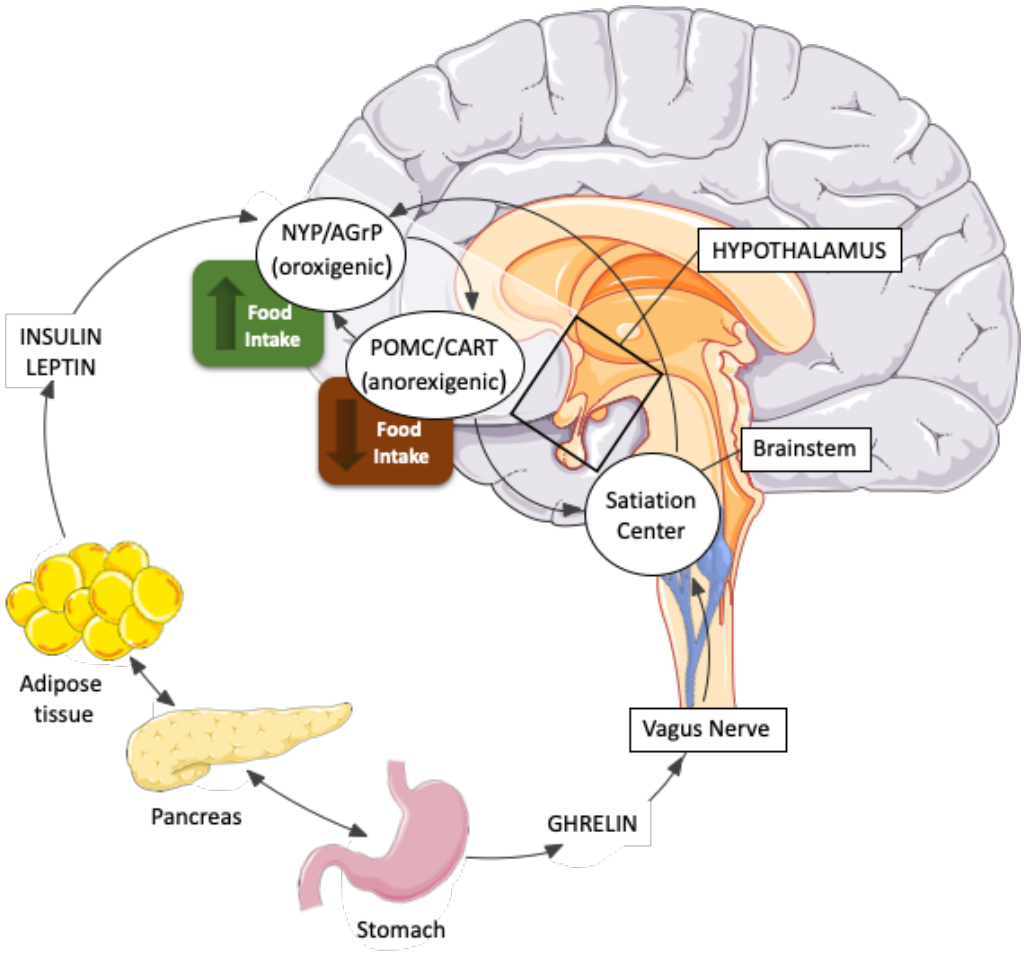


Figure 1.1 Hormonal and neuronal interactions in food intake regulation. Hormonal signaling includes insulin, leptin and ghrelin segregated by the pancreas, adipose tissue and stomach, respectively. In the hypothalamus there are orexigenic (NYP/AGrP) and anorexigenic (POMC/CART) neurons that regulate food intake. When insulin, leptin and ghrelin binds to orexigenic neurons food intake will increase. Anorexigenic neurons activated will inhibit eating behaviour. This figure was created using tools from *https://smart.servier.com.*

1.3 Non-Homeostatic regulation of food intake

Dopamine in the brain

Dopamine (DA) is the most abundant catecholamine neurotransmitter, corresponding to 80% of the catecholamine content in the brain. It acts in the basal ganglia, modulating several behavioural responses, including food related reinforcing responses (Vallone et al., 2000).

The synthesis of this neurotransmitter originates in the precursor aromatic amino acid L-tyrosine, which in the presence of the enzyme, tyrosine hydroxylase is converted into L-3, 4-dihydroxyphenylalanine (L-DOPA)**.** L-DOPA is decarboxylated to form dopamine (Cooper, 2003). This last step occurs in the presence of the enzyme, aromatic L-amino acid decarboxylase (AADC). Within neurons dopamine produced is stored in the presynaptic vesicles. When an action potential arrives at the nerve terminal, the synaptic membrane depolarizes causing an influx of calcium into the membrane, as a result, the vesicles fuse with the membrane, releasing dopamine to synapse. Once dopamine is released into the synaptic cleft, it only plays action when it is coupled to specific membrane receptors present at the post-synaptic terminal of another neuron. On the basis of biological and pharmacological properties, five dopamine receptors have been isolated and classified into D1-like and D2-like receptors (Missale et al., 1998; Vallone et al., 2000). The D1-like family includes D1 and D5 receptors, and the D2-like family includes D2, D3, D4 receptors. These receptors belong to a family of seven transmembrane domain G-protein coupled receptors. D1 -like receptors activate adenylyl cyclase and D2-like receptor inhibits adenylyl cyclase and Ca2+ channels and activate K+ channels. (Missale et al., 1998).

The most abundantly expressed DA receptors in the brain are the D1 and D2. D1 receptors are only expressed in postsynaptic neurons, such as, striatum, olfactory tubercle, cerebral cortex and amygdala. (Kim et al., 2015; Missale et al., 1998; Vallone et al., 2000). The D2 receptors are expressed mainly in striatum, olfactory tubercle, and also in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) , which are two of the regions that give origin to dopaminergic neurons, meaning that these receptors have not only postsynaptic but also presynaptic location. (Kim et al., 2015; Missale et al., 1998; Vallone et al., 2000). Once dopamine is released into the synaptic cleft it binds to specific D1 or D2 receptors depolarizing the consequent neuron.

The intensity and duration of dopaminergic signalling is therefore dependent on the inputs to the VTA and SNc, leading to the activation of dopamine neurons. The number of receptors at the post-synaptic neuron are also dependent on the rate at which dopamine is re-uptaken from the synaptic cleft into the presynaptic nerve terminals. Dopamine can be recycled and transported from the cytosol back into the vesicles, a mechanism mediated by the plasma membrane dopamine transporter (DAT) (Torres, 2006). Therefore, VTA or SN dopamine neuronal activity, D1 and D2 receptors availability and DAT activity are all responsible for the regulation of extracellular dopamine concentrations, influencing directly dopaminergic effects at presynaptic and postsynaptic level.

Structure and function of the dopamine system

Dopamine is synthetized in dopaminergic neurons, located in the midbrain nuclei englobing VTA and SNc. The VTA neurons projects to limbic (nucleus accumbens, amygdala and hippocampus) and cortical regions (prefrontal cortex, cingulate gyrus, temporal pole), establishing the mesolimbic and mesocortical pathways, respectively. The release of dopamine from the mesolimbic pathway into the

nucleus accumbens regulates motivation and desire for rewarding stimuli; and facilitates reinforcement and reward-related motor function learning (Wise, 2002). The SNc neurons projects to striatal regions (nucleus accumbens and dorsal striatum (caudate putamen)), assuming the nigrostriatal pathway, where the primarily function of dopamine is associated with motor control, like movement initiation (Volkow et al., 2013). Figure1.2. illustrates the different dopaminergic pathways.

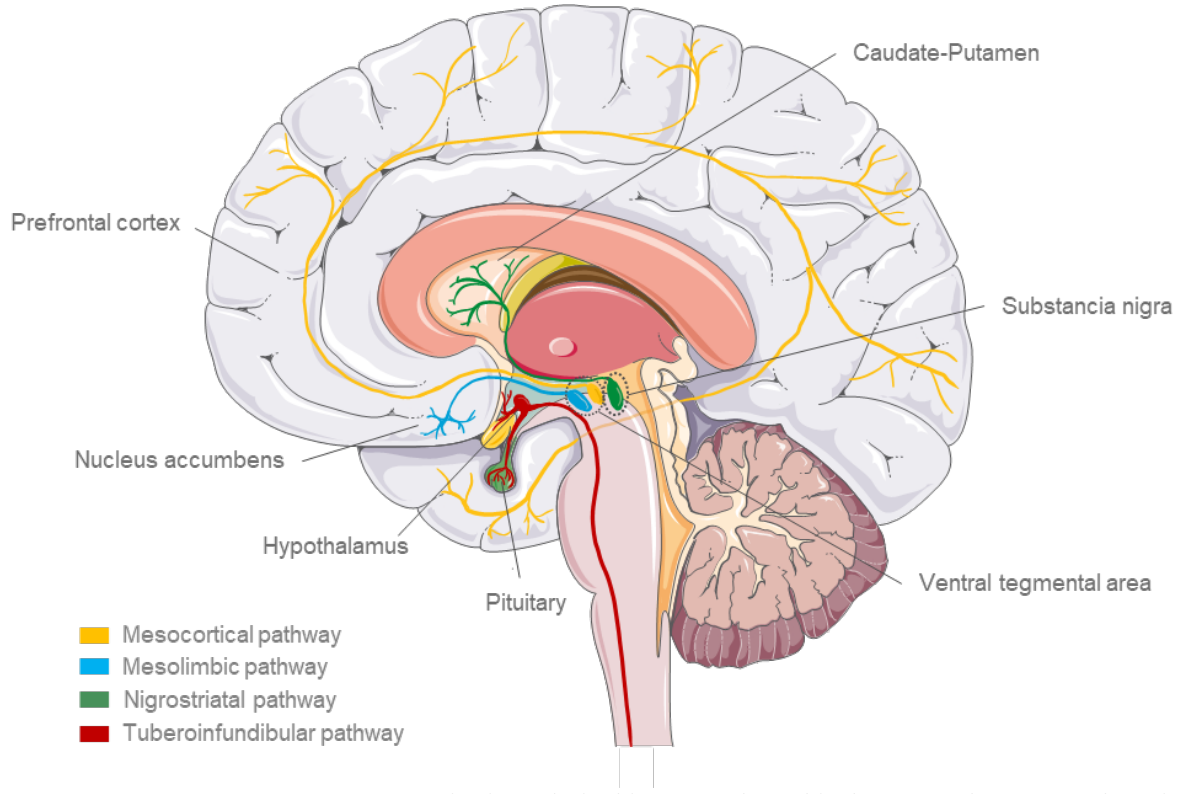


Figure 1.2. Dopaminergic pathways. Dopamine is synthetized in neurons located in the VTA and SNc. VTA dopamine neurons projects to nucleus accumbens and prefrontal cortex, establishing the mesolimbic (blue) mesocortical (yellow) pathways, respectively. SNc dopamine neurons project to the striatum, establishing the nigrostriatal pathway (green). The hypothalamus neurons projects to the median imminence, establishing the tuberoinfundibular pathway (red) This figure was created using tools from *https://smart.servier.com.*

The role of dopamine in reward and motivation

Reward is a term used for any stimuli, action, event, or object that prioritize behaviour, causing the animal to put effort in choosing and getting the reward, due to the pleasurable feeling properties that cause positive reinforcement (Schultz, 2015). In nature, we can find rewarding properties in natural pleasurable experiences such as food and sex, however, several studies have found that drugs of abuse, social reinforcers and brain stimulation have also such rewarding effects (Berridge and Robinson, 1998; Di Chiara and Imperato, 1988). This mechanism seems to be mostly mediated through the mesolimbic pathway (Volkow et al., 2008a).

The brain reward system corresponds to the neural circuits that regulate several aspects such as reward/incentive saliency, motivation, learning/conditioning, inhibitory control and executive function. (Volkow et al., 2008a; Wise, 2006). When linked to reward, motivation becomes a more complex concept, depending on factors such as the experience or habit strength, the drive state or duration of deprivation and the rewarding value of the object (Figlewicz Lattemann and Benoit, 2009). In the context of eating behaviour, if we consider factors such as the pleasure experienced during food

consumption, easy access to highly palatable and caloric food, the motivation to ingest food will be extremely high overruling the 'homeostatic control' of food intake (Figlewicz Lattemann and Benoit, 2009; Saper et al., 2002).

According to the incentive salience hypothesis a reward can be dissociated into two different components such as the 'liking' and 'wanting'. While 'liking' is linked to the hedonic value, or pleasurable feeling (e.g. palatability, associated with food), 'wanting' (or incentive salience), represents a desire that will stimulate the seek for that pleasurable object (e.g. food), and this is often considered as motivation (Berridge, 1996; Volkow et al., 2011). 'Wanting' is mostly measured by behaviour itself, on the other hand, 'liking' is much harder to measure due to its subjectivity/feelings. For instance, in animal experiments, 'wanting' is measured with a 'progressive ratio schedule of reinforcement' (Richardson and Roberts, 1996) that demonstrates how much effort an animal is willing to spend for a food reward, often by progressively increasing the amount of levers that needs to be pressed for each subsequent reward. Liking in animals is often measured by orofacial reactions. In reality, most of the time these terms overlap and depend on each other, because in fact, the more we like something, the more we want it.

There are several evidences showing that rewarding actions evokes an increase in DA activity, emphasizing that dopamine transmission is crucial for the reward system and associated behaviour functions. In human studies, electrical stimulation of the NAc led to a strong feeling of pleasure (Bishop et al., 1963). In addition, PET and MRI imaging studies reported that when humans were presented with addictive rewards such as drugs of abuse and video games, a modulation of activity in dopamine target regions such as striatum and prefrontal cortex was observed (Berridge and Robinson, 1998). In animal studies it was revealed that drugs of abused increased levels of dopamine in the NAc (Di Chiara and Imperato, 1988; Imperato and Di Chiara, 1986), and/or administration of antagonists drives to a reduction of consummatory behaviour.

The role of dopamine in food reward

Palatable foods show rewarding and pleasurable properties which drives excessive consumption. Dopamine is known to be important in conditioning to food stimuli as well as to regulate appetitive and food-motivated behaviors (Wang et al., 2002). As an example, it was reported that ingestion of palatable foods promotes dopamine release in the NAc and dorsal striatum, regions that are related to primary reward and motivation (Kelley, 2004; Martel and Fantino, 1996; Small et al., 2003).

Daily, humans are confronted with many cues related to food, such as advertisement, which also creates impact in food consumption. Unconsciously associated cues such as smell or sight can intensify food wanting (Peciña and Smith, 2010). Human studies in healthy women revealed that while watching fattening foods photographs, this actually increases significantly the activation of dopaminergic signaling in several brain areas (Schur et al., 2009). In animal studies, access to palatable foods led to activation of the dopaminergic signaling in striatum, NAc, amygdala (Ángeles-Castellanos et al., 2007).

Feeding behaviour and food preference is modulated not only by orosensory factors (palatability and environmental food cues) but also by post-ingestive signals (signals that occur after food has been swallowed) (De Araujo et al., 2013; Sclafani, 2001). Although palatability and post-ingestive effects might act together (Sclafani, 2001), in the last years the importance of the post-ingestive mechanisms emerged as important modulators of feeding behaviour. Several studies demonstrated that mice, rats and humans have preference for flavours paired with nutrients to flavours without any caloric content (de

Araujo et al., 2008; De Araujo et al., 2013; Touzani et al., 2008). Also, post-ingestive mechanisms are associated to increase in dopamine in mesolimbic areas, normally areas associated to reward (de Araujo et al., 2008; Mccutcheon, 2015; Touzani et al., 2008). Nevertheless, it is still unknown how these signals influence the motivation for food and if they could be involved in food disorders, such as obesity. Post-ingestive signals have been extensively studied in the context of behaviour feeding paradigms, using a transgenic mouse line with deficits in orosensory processing of sweet taste: the TRPM5 KO mice. This mouse has diminished peripheral neural responses to sweet tastants and does not show a preference for sweet tasting solutions in behavioral paradigms that are dependent on orosensory responses (Zang et al., 2003). Nevertheless, after conditioning, these mice develop a preference for caloric solutions, indicating learning based on post-ingestive properties, independently of orosensory feedback (Araujo et al., 2008; Sclafani and Ackroff, 2015). Post-ingestive mechanism can also be isolated by introducing directly into the stomach an intragastric catheter (Ueno et al., 2012) enabling the infusion of reinforcers directly into the stomach. Using this approach several authors have reported preferences for flavours paired with intragastric calories solutions over flavors paired with non-caloric solutions (Zukerman et al., 2013a, 2013b; Zhang et al., 2018).

Dopamine and obesity

Dopamine is a crucial neurotransmitter that is known to regulate food intake by modulating appetitive motivational processes via the reward pathway (Wise, 2006). Dysfunction of the dopamine reward system has been implicated in obesity. Reduced availability of dopamine 2 receptors (D2R) in the striatum has been found in obese animals and humans (Volkow et al., 2008b; Wang et al., 2001, 2009). Furthermore, knockdown of striatal D2 receptors resulted in increased appetite, inducing compulsive food craving behaviour and resulted in significant weight gain (Johnson and Kenny, 2010; Wang et al., 2001). Thus, excessive overconsumption of palatable foods and the development of obesity appear to trigger addiction-like neuroadaptive responses in the striatal dopamine system that may contribute to the development of compulsive eating.

Besides the evidence showing impairments in dopamine activity in obesity there is no causal correlation between dopaminergic impairment and obesity onset. The main hypothesis on correlations and biological data either in mice, rats and humans suggest that dopamine deficiency lead to overconsumption of highly rewarding food rich in fat and sugar in a way to compensate for this deficiency (Blum et al., 1996, 2000; South et al., 2012). This dopamine deficiency hypothesis may explain preference for this type of food by many obese individuals.

Taken together, understanding orosensorial and post-ingestive responses during food consumption and their impact within the dopaminergic system is one of the main focus of this thesis. How those mechanisms lead to overconsumption and the development of obesity is the second main objective of this thesis.

Aims

The main objective of this thesis was to understand how food stimuli modulate the ventral tegmental area (VTA) dopaminergic activity. While there is clear evidence that both early (oral) and late (post-ingestive) reinforcing effects of food consumption contribute significantly to modulate food intake (de Araujo et al., 2008; Sclafani et al., 2015), fundamental mechanistic aspects of this process, such as how post-ingestive signals are integrated in the brain, are still unresolved.

Another question regards responses of post-ingestive reinforcement to different reinforcers. Unpublished data from Oliveira-Maia Lab have shown that VTA dopamine neurons increase significantly their activity when sucrose is infused directly into the stomach (compared to a sweet non-caloric solution). But the specificity of this response is still unknown. To investigate this issue, we proposed to develop 3 specifics aims:

Aim 1: VTA dopamine neuron's response to different intragastric infusion of nutrient

Here, we wanted to investigate if the VTA dopaminergic neurons respond to different post-ingestive nutrient reinforcers (carbohydrate, fat and protein), and if it does, if that response will be nutrient specific. Our hypothesis was that VTA dopamine neurons increased their activity in a nutrient specific manner, responding differently to carbohydrates and fat.

In order to obtain some answers for these questions, an innovative approach was used, where the activity of VTA dopaminergic neurons was measured during the intragastric delivery of the different reinforcers according to their nutrient (carbohydrate, fat and protein). Before measurements of VTA dopaminergic activity mice were submitted to several procedures (for more detailed information see Materials and Methods). This task was conducted in different days, two sessions for each reinforcer and a control session where a reinforcer was infused without any nutritional value (an artificial sweetener was used: sucralose).

Aim 2: Determine if VTA dopaminergic activity is altered by diet-induced obesity

Aim2.1: Identify diets capable of promoting significant weight gain.

It has been reported that obese patients have a reduced density of dopamine D2 receptors which has been related to compulsive food intake and weight gain (Johnson and Kenny, 2010). However, the importance of dopamine synthesis and dopamine neuron activity in the development of obesity is mostly unknown. We thus intend to explore how VTA dopaminergic activity is modulated by food stimuli and by weight variations. Specifically, we proposed to explore, during the onset and development of obesity how dopaminergic responses to food rewards are modulated.
We hypothesized that dopaminergic response to food rewards were modulated as obesity develops. To address this hypothesis, we first needed to induce obesity in mice, and that was the second specific aim of this thesis: define the best diet to induce obesity during a 12-week period. Our hypothesis is that diet manipulations through high-carbohydrate diets or High-Fat diets promote weight gain.

Several diets were tested, such as High-Fat and High-Carbohydrate solid diets (pellets) and a 35% sucrose solution (where the regular chow continued to be available) was also tested. Along 12 weeks

weight gain, food and water consumption were measured and always compared to a normal standard diet.

Aim 3: Determine if post-ingestive feedback is sufficient to induce weight changes.

In this third aim we used a genetical knock-out animal model where a taste TRP ion Channel was knocked-out. In this strain model sweet taste reception is abolished in homozygotes (Zhang et al., 2003). Previous results have shown that Trpm5 KO mice develop a preference for sucrose after a flavour-nutrient conditioning protocol indicating a post-ingestive mechanism that defines food preference independent of orosensorial component. We decided to study diet-induced obesity in this animal model in order to reveal if orosensorial component was an important feature in weight gain. With this aim and extrapolating that in this animal model the major feeding rewarding mechanism is the post-ingestive feedback, we aim to understand if post-ingestive stimulation is sufficient to induce weight gain. Trpm5KO and respective littermates were exposed to 35% sucrose solution (where the regular chow continued to be available) along 12 weeks. Weight gain, food consumption and sucrose/water consumption were measured along 12 weeks.

2. Materials and Methods

All experiments were approved by the Champalimaud Foundation and Portuguese *Direção Geral de Veterinária* and done in accordance with European Union Directive for Protection of Vertebrates Used for Experimental and other Scientific Ends (86/609/CEE).

Animals

In this work, different mice strains were used, namely, C57BL/6J (B6); 129-Trpm5^{tm1Csz}/J (Tprm5), B6.SJL-Slc6a3$^{tm1.1(cre)Bkmn}$/J (Dat-cre). With exception of the Dat-cre mice all the other strains were inbred at the Champalimaud foundation vivarium. Dat-cre were obtained from The Jackson Laboratory®.

The C57BL/6J strain is the most widely used and is sensitive to the effects of diet on weight, being a particularly good model of the human metabolic syndrome because when raised on a high-caloric ad libitum diet, it develops severe obesity, hyperglycemia and hyperinsulinemia (Surwit et al., 1995).
The B6;129-Trpm5^{tm1Csz}/J strain carries a targeted mutation of the Trpm5 (transient receptor potential cation channel, subfamily M, member 5 gene which encodes a taste TRP ion Channel. In this strain model, sweet, amino acid and bitter taste reception is abolished in homozygotes (Zhang et al., 2003).The B6.SJL-Slc6a3$^{tm1.1(cre)Bkmn}$/J, also known as DAT-Cre/J, is a transgenic line that expresses cre-recombinase in VTA under the transcriptional control of the endogenous DAT promoter, enabling, without disrupting endogenous dopamine transporter, the expression of floxed genes specifically in VTA dopaminergic neurons (Bäckman et al., 2006). Genetically defined neurons that express cre-recombinase can reliably be targeted for imaging in anatomically defined brain regions in adult animals by utilizing cre-inducible viral constructs that are stereotaxically delivered to the brain region of interest, in this case the VTA. Also, the viral expression in this mice line is restricted to VTA DAT-positive neurons. (Stuber et al., 2015). This approach gives region specificity that is necessary for tracking dopaminergic VTA activity.

All the animals were housed individually in a temperature-controlled room with a 12-hour light/dark cycle, and they were allowed to ad libitum access to water and food.

Nutrient solutions and Diets

Three different nutrient solutions were tested. A fat solution of 9% corn oil (Sigma-Aldrich, Missouri, USA), dissolved in 0,4% Emplex® emulsion, a carbohydrate solution of 20% sucrose (Sigma-Aldrich, Missouri, USA) and a protein solution of 20% L-glutamic acid solution (Sigma-Aldrich, Missouri, USA). All the concentrations were calculated in order to obtain isocaloric solutions, having as reference the 20% sucrose solution. The 20% sucrose solution was based on previous studies performed within the lab (unpublished data) showing that this concentration is enough to induce behavioral post-ingestive reinforcing properties and induce significant activation of VTA dopamine neurons. For controlling caloric content and gastric emptying effects associated to the intragastric infusion a sweet non caloric solution of 0,02% sucralose was used. Sucralose was also an important control solution since sweet receptors have been described within the intestine (Yamamoto and Ishimaru, 2013), and therefore it was important to exclude sweet receptor activation as one of the major post-ingestive mechanism.
In order to test the best diet to induce obesity in mice during 12 weeks, a liquid 35% solution was *ad libitum* available in mouse home cage. Two other solid diets were also tested, a High-Fat Diet (HF)

(Envigo, MD.06414 60% (Kcal) fat, 18.3% (Kcal) protein and 21.7%(Kcal) carbohydrate) and a High-Carbohydrate Diet (HC) (Envigo, MD.1806 70% Carbohydrate, 17.8% protein and 11.8% fat). For the control groups, a standard diet, Envigo, 914 Teklad ®, was used (67% Carbohydrate, 13% protein and 20% fat. All diets were purchased from Envigo®, United Kingdom. For Additional nutritional information see figure 2.1 and 2.2.

Teklad Custom Diet

TD.06414

Adjusted Calories Diet (60/Fat)

Formula	g/Kg
Casein	265.0
L-Cystine	4.0
Maltodextrin	160.0
Sucrose	90.0
Lard	310.0
Soybean Oil	30.0
Cellulose	65.5
Mineral Mix, AIN-93G-MX (94046)	48.0
Calcium Phosphate, dibasic	3.4
Vitamin Mix, AIN-93-VX (94047)	21.0
Choline Bitartrate	3.0
Blue Food Color	0.1

Footnote

Approx. 60% of total calories come from fat. Designed with similarities to Research Diets, Inc. formula D12492. For the series TD 06414-TD 06416. Approximate fatty acid profile (% of total fat): 37% saturated, 47% monounsaturated, 16% polyunsaturated.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	23.5	18.3
Carbohydrate	27.3	21.4
Fat	34.3	60.3
Kcal/g 5.1		

[1] Values are calculated from ingredient analysis or manufacturer data

Figure 2.1. Nutritional composition of the 60% High-Fat MD.06414 Envigo® diet.

Teklad Custom Diet

TD.98090

70% Carbohydrate Diet

Formula	g/Kg
Casein	200.0
DL-Methionine	3.0
Sucrose	645.6
Corn Starch	20.0
Maltodextrin	20.0
Soybean Oil	50.0
Cellulose	9.89
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.5
TBHQ, antioxidant	0.01
Mineral Mix, AIN-93G-MX (94046)	35.0
Calcium Phosphate, dibasic	4.0

Footnote

A diet with 70% carbohydrate as expressed by weight and % of total kcal. Sucrose makes up approximately 66% of the diet (includes contribution from premixes).

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	17.7	17.8
Carbohydrate	70.0	70.4
Fat	5.2	11.8
Kcal/g 4.0		

[1] Values are calculated from ingredient analysis or manufacturer data

Figure 2.2. Nutritional composition of the 70% High-Carbohydrate MD.1806 Envigo® diet.

Surgical procedure for viral injection, lens implantation and intragastric catheter placement

Mice were anesthetized throughout the surgery with a mix of isoflurane (1-3%) and oxygen (1-1.5l/min). Mice were placed on the surgical table with a heating pad (37ºC). The hair was cut, and the skin and muscle layer were clipped in the abdomen or in the skull with the aid of surgical instruments (scissors and tweezers). Surgical interventions were performed with the addition of swabs, gauzes and sutures.

Gastric catheter implantation. intragastric solutions were administered directly in the stomach, through a catheter (Instech Solomon) placed into the stomach and exteriorized through the skin and the muscle layer. The catheter tips were placed in the upper part of the stomach and the other part in the dorsum. A small incision in the dorsum was performed, allowing the exteriorization of the catheter. Incisions were closed using absorbable and non-absorbable suture lines. In all procedures aseptic conditions were kept and all the material was carefully washed and sterilized before each surgical procedure. After surgery the animal was placed in a clean home cage and recovered in a heating pad. Postoperative analgesia was administered whenever necessary.

Viral Injections, GRIN lens implantation and baseplate fixation. The head of the animals were fixed with a stereotaxic system, allowing the fixation and alignment of the head of the mouse. The skull of the animals was exposed through an incision across the skin, To ensure skull was leveled, the height of bregma in relation to lambda was compared (difference in mean <0.05mm) and lateral distance (+/- 2.0mm) from bregma was also confirmed (difference in mean <0.05mm). Bregma coordinates were used as a guidance for the coordinates of VTA (Antero-Posterior: -2.98mm and Dorso Lateral: ±0.4mm and from bregma), accordingly to a standardized atlas of the brain "The Mouse Brain in Stereotaxic Coordinates" (Franklin, B.J., and Paxinos, G., 2008), see figure 2.3.

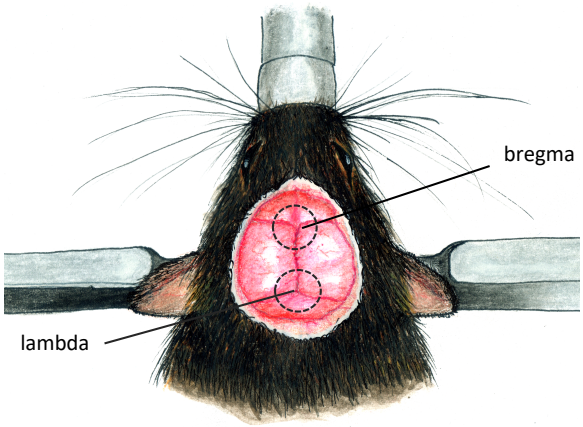
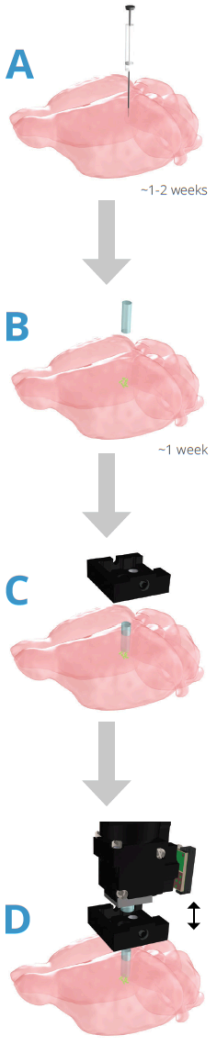


Figure 2.3. Bregma and lambda. reference points to calculate stereotaxically coordinates of brain regions



Using a glass pipette, unilateral viral injection of GCaMP6f virus (AAV5.CAG.Flex.GCaMP6f.WPRE.SV40 - UPENN) was performed to target VTA dopamine neurons. 1μl GCaMP6f viral was injected with help of a micro pump device, Nanojet II (Drummond Scientific Company, Broomall, USA), attached to the stereotaxis arm. 4,6 ηl virus was injected every 5 seconds, along 18 minutes, see figure 2.4 (A). Considering these steps, it is important to highlight that the virus must be preserved in ice and free from light, since this is as fluorescent sensor which the fluorescence decays during light exposure and, at room temperature. A GRIN lens was afterwards implanted in the VTA. Before lens (GRIN lens) implantation, skull screws were implanted in two holes drilled, to support the head cap. For lens implantation, a lens holder was attached to the stereotaxis arm. Then, like it was done in the previous step, the lens was lodged in the cortex and introduced, according to the coordinates, along the brain until reached the VTA, however due to the lens size (0.5x8.4mm) this procedure was performed slowly, due to the possibility of dragging cortex material with the lens further damaging the image, see figure 2.4(B). GRIN Lens (nVistaHD, Inscopix) was fixed using superglue and dental cement (Contemporary Ortho-Jet™ Liquid and Powther, LANG). Dental cement was also used to create a head cap covering the skull screws and part of the lens. A temporary adhesive tape was placed on top of the head cap to prevent damage of the lens. After 4 weeks a baseplate was attached using cement to the head cap above the GRIN lens allowing the connection and disconnection of a miniaturized microscopes that will be used during imaging of VTA dopaminergic neurons protocol (Resendez et al., 2016), see figure (C,D).

Figure 2.4. Workflow methods for implanting a lens probe and installing nVista. Viral injection (A); GRIN lens implantation (B); baseplate application (C); mini endoscope attachment. Image from "Deep brain imaging with Nvista™", application highlight, inscopix®

After a recovery period, food deprived mice were submitted to six sessions in different days, each two for the same nutrient (which means, 2 days for fat, 2 days for sucrose, 2 days for protein). In each session the mouse (connected to the mini endoscope) was placed in chambers housed within sound attenuating boxes, with a solution dispenser connected to the gastric catheter and a video camera for tracking animal movement. The session started with a five minutes baseline; period preceded by the infusion of the reinforcer directly into the stomach. The reinforcer was infused along 90 seconds and VTA dopaminergic neurons were image continuously during the 30 minutes, including the 5 min baseline and the intragastric reinforcer infusion.

VTA Dopaminergic calcium imaging Activity

In neuroscience, the limitations of conventional microscopy difficult the scope and scale of brain-imaging studies, making it impossible to perform experiments in freely behaving mice, while tracking neuronal activity in vivo (Resendez et al., 2016). However, to break some of these challenges an innovative technology was developed, which is a miniature, integrated fluorescence microscope, enabling simultaneous imaging of individual neurons, while preserving cellular level resolution and while animals are behaving freely.

Acquisition of in vivo Ca2+ imaging data during intragastric nutrient delivery

4 weeks after viral injection, baseplate placement and VTA dopaminergic neurons visualization, the intragastric infusion of isocaloric solutions started. Food-deprived mice with a GRIN lens fixed in the VTA and an implanted intragastric catheter were rapidly anesthetized with isofluorane to allow for the attachment of the microscope and placement of the catheter for the intragastric administration of different solutions. A miniature microscope (nVista HD, Inscopix) was attached to the baseplate and a circuit for flashing light emitting diode (LED) was linked and synchronized to the session start. Mice were placed in the recording chamber and were allowed to move freely during the imaging of GCaMP6f fluorescence of VTA dopamine neurons. Each session was performed after a 30 min acclimatization period, to allow full recovery from anesthesia. Fluorescence images were acquired using nVista acquisition software (Inscopix) at 10 Hz with nVista HD light-emitting diode power at 30%. These features were adjusted for each mouse and kept constant across different sessions. The imaging session was initiated by a baseline period with no stimulation during 5 min, followed by 90 sec period where intragastric solutions where infused directly into the stomach. The session was run during 30 min to avoid photobleaching. Each mouse was recorded daily for a few consecutive days. At the end of each experimental procedure, the microscope was disconnected, and baseplate cover was attached to the baseplate.

Imaging data processing

Data were imported and processed by Mosaic analysis software (Inscopix) and Matlab2015A, accordingly to the Inscopix user guide (Inscopix Data Processing Software User Guide, Release 1.2.1, 2019). Each session movie was down sampled and soothed for motion artefacts. Motion correction was employed using a single frame as a reference frame. Once it was processed, an automated Ca2+ sorting using the Constrained Nonnegative Matrix Factorization for endoscopes (CNMF-e) algorithm (Klaus et al., 2017) was used, allowing the identification of cell location and activity.

Weight, water and food consumption parameters acquisition

The weight was measured on a weight scale and a container to keep the mouse on the top of the balance. Mice were kept on a container and weight was measured. To obtain the weight difference, first the average weight of each mouse in the accommodation period was calculated, and then it was calculating through the subtraction between the absolute weight (in each day, during the diet manipulation) and the average weight.
For the calculation of pellet consumption, in each day the pellet was measured on a weight scale and registered, then, the consumption was obtained by subtracting the pellet weight of the previous day with the pellet weight of the current day.
Along the DIO protocol, an empty cage was added in the same place were the animals were placed for measurement of residual water loss. This residual water loss was obtained by the difference of the water amount in each day compared to the previous one. The amount of water consumed was calculated by the difference of the total water volume in the bottle of each day with the respective previous day. Finally, the real water consumption was the difference between the amount of water consumed and the residual water loss in each day.
The units for each measure were grams (g) for the weights and milliliters (ml) for the volumes. All the data above mentioned were registered and calculated using the software program Microsoft Excel®.

Statistical analysis

Results were presented mean ± standard error of mean (SEM) and statistical significance was accepted for $p<0.05$. Statistical analysis was conducted using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) and Matlab using statistical toolbox (The MathWorks Inc, MA). Two-way ANOVAs were used to investigate main effects; and post-hoc comparisons (Bonferroni post-hocs) where performed whenever appropriate.

3. Results

Aim 1: VTA dopamine neurons response to different intragastric infusion of nutrients

We propose to understand the relationship between VTA dopaminergic neuron activity and intragastric delivery of different nutrients such as fat, carbohydrate and protein. Three different isocaloric solutions were tested: a 9% corn oil, 20% sucrose and 20% glutamate. A 0,02% sucralose was used as control for gastric distension. The solutions were intragastric (IG) delivered through an intragastric catheter previously inserted in the stomach, while and simultaneously the VTA dopaminergic neuron activity was recorded. Figure 3.1. shows the mean fluorescence activity of VTA dopaminergic neurons during the injection of the three different nutrients previously described.
The figure 3.1. shows 5 minutes before and 10 minutes after reinforcer infusion. The profiles of all the reinforcers tested showed the most significant alteration in activity occurred immediately after reinforcer delivery into the stomach. 10 minutes after infusion the VTA dopaminergic activity was back to the baseline values. VTA dopamine neurons increased activity when sucrose was infused into the stomach Other isocaloric reinforcers did not show this effect.

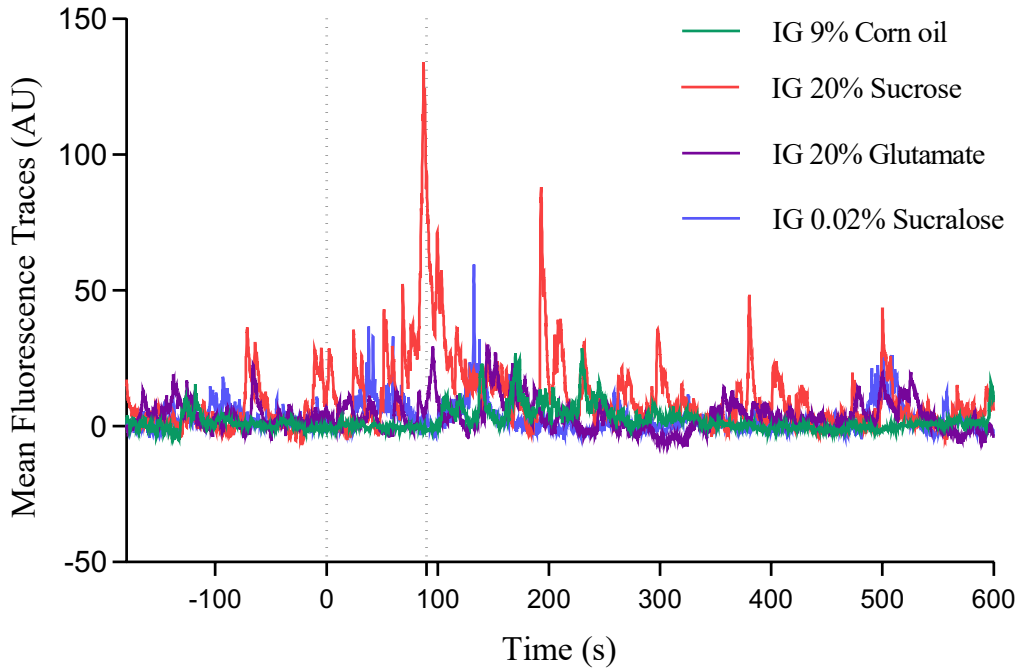


Figure 3.1. Results of VTA dopamine neurons activity recorded during intragastric infusion of different nutrient solutions. The graphic represents the mean of VTA neuronal fluorescence detected in two sessions for each nutrient solution, 9% corn oil (green), 20% sucrose (red), 20% glutamate (purple) and 0,02% sucralose as the non-caloric control. The data is presented in raw format and correspond only to one mouse (n=1). For both sessions we could record 7 neurons for intragastric sucrose intragastric infusion; 8 neurons for intragastric corn oil; 6 neurons for intragastric glutamate and 11 neurons for sucralose intragastric infusion.

Comparing the mean activity of VTA dopaminergic neurons before and 5 minutes after injection of each intragastric reinforcer we observed that all the reinforcers increase the overall mean activity of these neurons, after intragastric injection (probably in response of stomach distension). However, sucrose intragastric infusion was the one that seem to have a more pronounced increase suggesting a nutrient specific response (figure 3.2.). The 5 minutes period selection was due to the imaging period encountered before the injection which was 5 minutes. Also, after this time period VTA dopamine activity was already superimposable between the reinforcers tested.

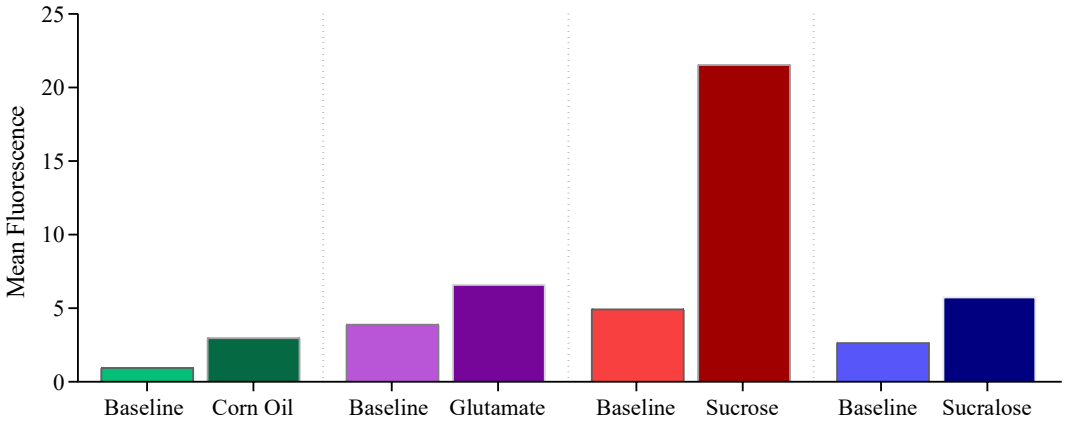


Figure 3.2. Results of VTA dopamine neurons activity before and after infusion of different nutrient solutions directly into the stomach. The graphic represents the mean fluorescence of all neurons detected in the two consecutive sessions of gastric infusion of each nutrient solution, 9% corn oil (green), 20% sucrose (red), 20% glutamate (purple) and 0,02% sucralose as the non-caloric control, in the baseline and after the infusions. Values correspond to the 5-minute baseline mean compared to 5 min of intragastric reinforcer delivery of each nutrient.

Figure 3.3. Results of weight and weight difference relative to the mean of one week prior to DIO exposure and during the HF vs. HC DIO protocol along 12 weeks. Weight (left) and weight difference (right) of animals submitted to HF diet (green squares, N=4) vs. HC diet (red. N=4) vs. control group (blue circles, N=6). A Two-way ANOVA was performed. **Left.** **p=0.0313; **p=0.0018; **Right**. ***p=0.0003; ****p<0.001.* **Figure 3.2. Results of VTA dopamine neurons activity before and after infusion of different nutrient solutions directly into the stomach.** The graphic represents the mean fluorescence of all neurons detected in the two consecutive sessions of gastric infusion of each nutrient solution, 9% corn oil (green), 20% sucrose (red), 20% glutamate (purple) and 0,02% sucralose as the non-caloric control, in the baseline and after the infusions. Values correspond to the 5-minute baseline mean compared to 5 min of intragastric reinforcer delivery of each nutrient.

Aim 2: Determine if VTA dopaminergic activity is altered by diet-induced obesity

We propose to study a causal approach to understand the relationship between VTA dopaminergic neuron activity and weight changes, in particular during the development of obesity. To induce weight gain three different diets were tested. 2 solid diets: A High-Fat Diet (HF) (60% Fat) and a High-Carbohydrate Diet (HC) (40% carbohydrates) and a liquid diet 35% sucrose solution. For the solid diets standard pellets were substituted by the high caloric pellets. For the liquid diet water was substituted by 35% sucrose solution.

2A. Solid diets
Effect of High-Fat and High-Carb diets on weight gain, food and water consumption

HF and HC were tested along 12 weeks. Figure 3.3. shows weight gain and weight difference when animals were exposed to HF and HC diets, respectively. HF diet increases significantly weight along the 12 weeks, reaching around 43 grams after 12 weeks. This effect is significant immediately 3 days after animals started HF diet exposure. As expected, animals on a standard diet did not show any significant increase in their body weight within the 12 weeks of protocol.
In the case of HC diet (figure 3.3.), the results don't show the same pattern as for HF diet. Mice exposed to HC diet have a superimposable profile to the control standard diet mice without any significant increase of weight at any timepoint of the 12 weeks of the protocol.

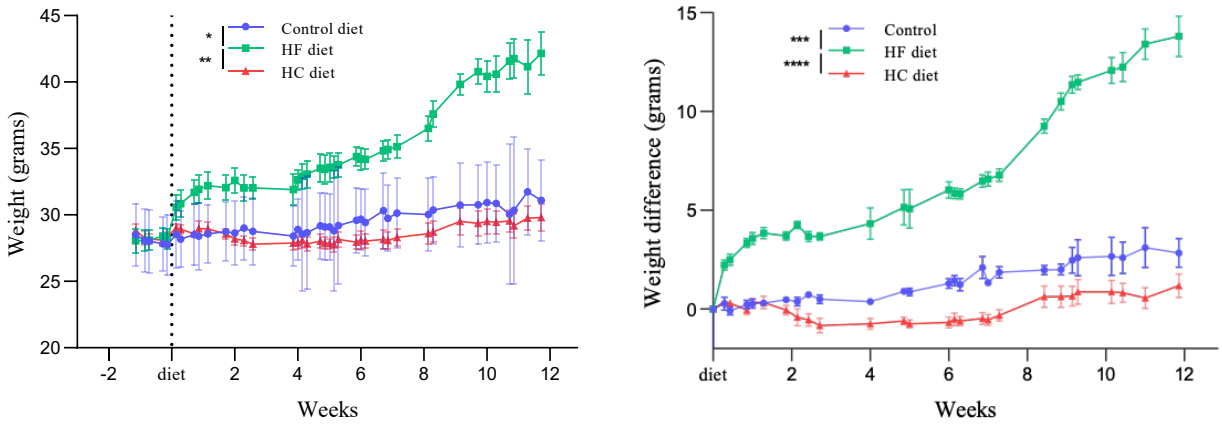


Figure 3.3. Results of weight and weight difference relative to the mean of one week prior to DIO exposure and during the HF vs. HC DIO protocol along 12 weeks. Weight (left) and weight difference (right) of animals submitted to HF diet (green squares, N=4) vs. HC diet (red. N=4) vs. control group (blue circles, N=6). A Two-way ANOVA was performed. **Left.** **p=0.0313; **p=0.0018; **Right**. ***p=0.0003; ****p<0.001.*

Figure 3.4. Results of pellet and the water consumption during the HF vs. HC diet DIO protocol along 12 weeks. Pellet consumption (left) and water consumption (right) of animals submitted to HF diet (green. N=4) vs. HC diet (red. N=4) vs. control group (blue, N=6). The solid bars correspond to the pellet and water consumption in the baseline (before starting the protocol), while the pattern bars correspond to the data after and during the introduction of test diets. A Two-way ANOVA was performed. **Left.** Baseline vs. control **p=0.0191*; control vs. HFD **p=0.0298*; baseline vs. HFD ****p=0.0006*; baseline vs. HCD ****p=0.0008*; **Right.** **p=0.0445*.**Figure 3.3. Results of weight and weight difference relative to the mean of one week prior to DIO exposure and during the HF vs. HC DIO protocol along 12 weeks.** Weight (left) and weight difference (right) of animals submitted to HF diet (green squares, N=4) vs. HC diet (red. N=4) vs. control group (blue circles, N=6). A Two-way ANOVA was performed. **Left.** **p=0.0313; **p=0.0018; **Right**. ***p=0.0003; ****p<0.001.*

In addition to measurements of body weight, food and water consumption for each diet was also measured. Figure 3.4. shows that animals independent of the diet decreased significantly food consumption. Exposure to HF pellets significantly reduced water intake when compared to mice exposed to control standard diet. Independently of the significant decrease in food and water intake mice exposed to HF diet increase significantly body weight suggesting that HF diets are efficient diets for diet-induced obesity.

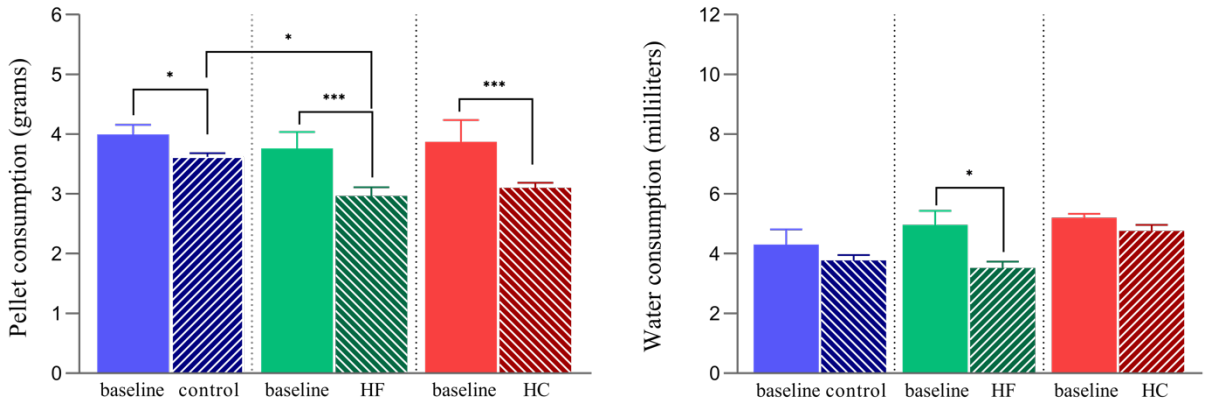


Figure 3.4. Results of pellet and the water consumption during the HF vs. HC diet DIO protocol along 12 weeks. Pellet consumption (left) and water consumption (right) of animals submitted to HF diet (green. N=4) vs. HC diet (red. N=4) vs. control group (blue, N=6). The solid bars correspond to the pellet and water consumption in the baseline (before starting the protocol), while the pattern bars correspond to the data after and during the introduction of test diets. A Two-way ANOVA was performed. **Left.** Baseline vs. control **p=0.0191*; control vs. HFD **p=0.0298*; baseline vs. HFD ****p=0.0006*; baseline vs. HCD ****p=0.0008*; **Right.** **p=0.0445*.

Figure 3.5. Results of weight and weight difference relative to the mean of one week prior to DIO exposure and during the 35% sucrose solution DIO protocol along 12 weeks. Weight (left) and weight difference (right) of animals submitted to a 35% sucrose solution (red squares N=7) vs. control group (blue circles, N=6). Two-way ANOVA was performed. **Left.** **p=0.0139;* **Right.** ****p=0.007.***Figure 3.4. Results of pellet and the water consumption during the HF vs. HC diet DIO protocol along 12 weeks.** Pellet consumption (left) and water consumption (right) of animals submitted to HF diet (green. N=4) vs. HC diet (red. N=4) vs. control group (blue, N=6). The solid bars correspond to the pellet and water consumption in the baseline (before starting the protocol), while the pattern bars correspond to the data after and during the introduction of test diets. A Two-way ANOVA was performed. **Left.** Baseline vs. control **p=0.0191*; control vs. HFD **p=0.0298*; baseline vs. HFD ****p=0.0006*; baseline vs. HCD ****p=0.0008*; **Right.** **p=0.0445*.

2B. Liquid Diets:
Effect of 35% sucrose diet on weight gain, food intake and water consumption

Inducing obesity using liquid diets was important since as a future approach we wanted to test the VTA dopamine response of that diet when infused the reinforcer of DIO directly into the stomach. A 35% sucrose solution protocol was started in another group of animals and as previously shown for the solid diets weight gain, food and water consumption was measured along 12 weeks.

Figure 3.5. shows that animals on a liquid sucrose diet increased significantly body weight when compared to animals exposed to water (same solid food in both groups).

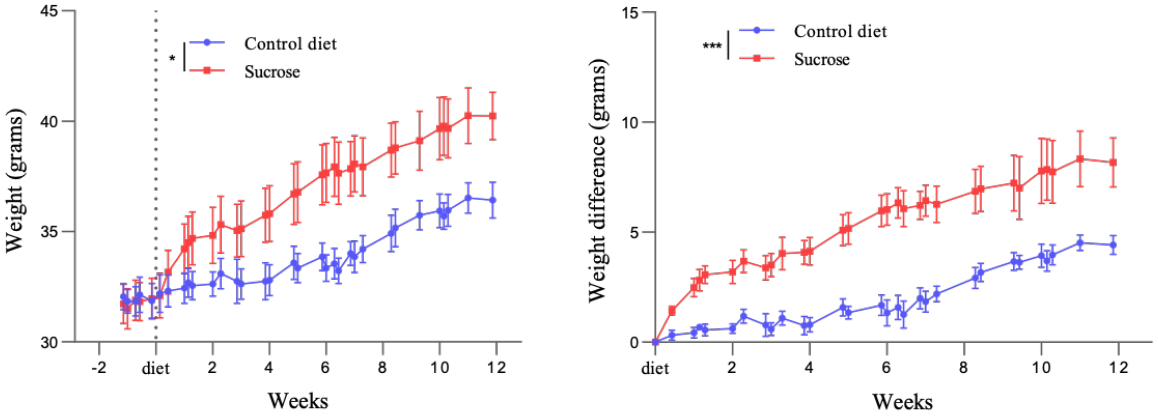


Figure 3.5. Results of weight and weight difference relative to the mean of one week prior to DIO exposure and during the 35% sucrose solution DIO protocol along 12 weeks. Weight (left) and weight difference (right) of animals submitted to a 35% sucrose solution (red squares N=7) vs. control group (blue circles, N=6). Two-way ANOVA was performed. **Left.** **p=0.0139;* **Right.** ****p=0.007.*

Figure 3.6. Results of pellet and the water consumption during the 35% sucrose solution DIO protocol along 12 weeks. Pellet consumption (left) and water consumption (right) of animals submitted to 35% sucrose solutions (red, N=7) vs. control (blue, N=6). The solid bars correspond to the pellet and water consumption during the baseline (before starting the protocol), while the pattern bars correspond to the data after starting the 35% sucrose. A Two-way ANOVA; *****p<0.0001*; **p<0.05*.

In this group of animal food and liquid consumption was also measured and it was observed that animals exposed to 35%sucrose solution decreased significantly food consumption but increase significantly the intake of the liquid sucrose solution when compared to animals that had access to water (figure 3.6.). The increase in sucrose consumption is probably responsible for the significant increased in body weight.

Figure 3.5. Results of weight and weight difference relative to the mean of one week prior to DIO exposure and during the 35% sucrose solution DIO protocol along 12 weeks. Weight (left) and weight difference (right) of animals submitted to a 35% sucrose solution (red squares N=7) vs. control group (blue circles, N=6). Two-way ANOVA was performed. **Left.** **p=0.0139;* **Right.** ****p=0.007.*

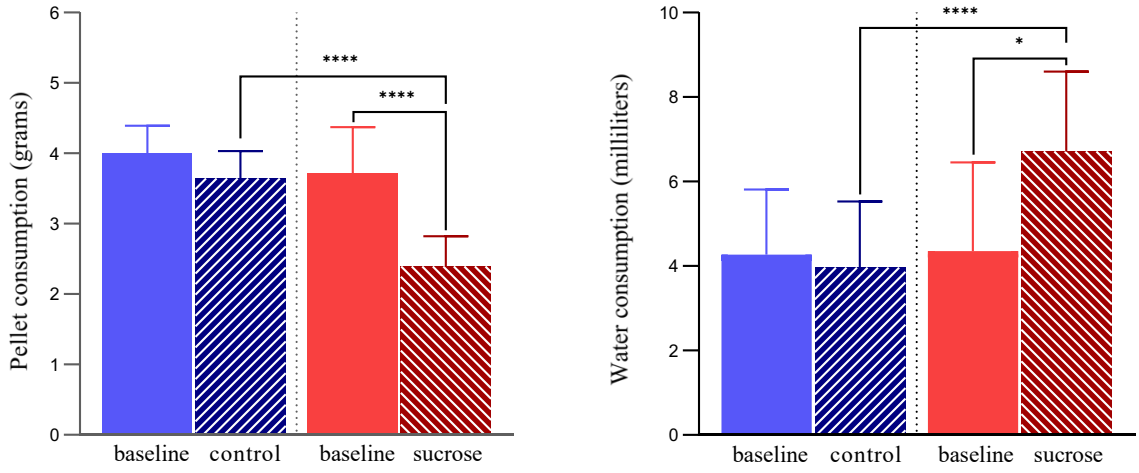


Figure 3.6. Results of pellet and the water consumption during the 35% sucrose solution DIO protocol along 12 weeks. Pellet consumption (left) and water consumption (right) of animals submitted to 35% sucrose solutions (red, N=7) vs. control (blue, N=6). The solid bars correspond to the pellet and water consumption during the baseline (before starting the protocol), while the pattern bars correspond to the data after starting the 35% sucrose. A Two-way ANOVA; *****p<0.0001*; **p<0.05*.

Figure 3.7. Results of weight in Trpm5 knock out mice (Trpm5) and control littermates (WT) mice relative to the mean of one week prior to DIO exposure and during the 35% sucrose solution DIO protocol along 12 weeks. Top. Weight(g) of Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4) vs. Trpm5 control (blue circles, N=5) and WT submitted to 35% sucrose solutions (orange triangles, N=7) vs. WT control (turquoise rhombus, N=4) **Right.** Weight(g) of Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4) vs. Trpm5 control (blue circles, N=5). **Left.** Weight(g) of WT submitted to 35% sucrose solutions (orange triangles, N=7) vs. **Figure 3.6. Results of pellet and the water consumption during the 35% sucrose solution DIO protocol along 12 weeks.** Pellet consumption (left) and water consumption (right) of animals submitted to 35% sucrose solutions (red, N=7) vs. control (blue, N=6). The solid bars correspond to the pellet and water consumption during the baseline (before starting the protocol), while the pattern bars correspond to the data after starting the 35% sucrose. A Two-way ANOVA; *****p<0.0001*; **p<0.05*.

Aim 3. Determine if post-ingestive feedback is sufficient for diet-induced obesity

In order to test the importance of post-ingestive feedback for the development of DIO genetically modified mice with absent of sweet taste transduction (trpm5-/- KO mice), as well as the respective wildtype (WT) littermate animals were exposed to a 35% liquid sucrose diet. Measurements of body weight, food and liquid solutions consumption were performed during the 12 weeks of protocol.
Figure 3.7. and 3.8. shows that Trmp5 mice exposed to a 35% sucrose solution significantly increased body weight when compared to Trpm5 mice exposed to water. This increase in body weight is similar to control littermates exposed to 35% sucrose, indicating that the lack of sweet receptors does not impact significantly DIO.

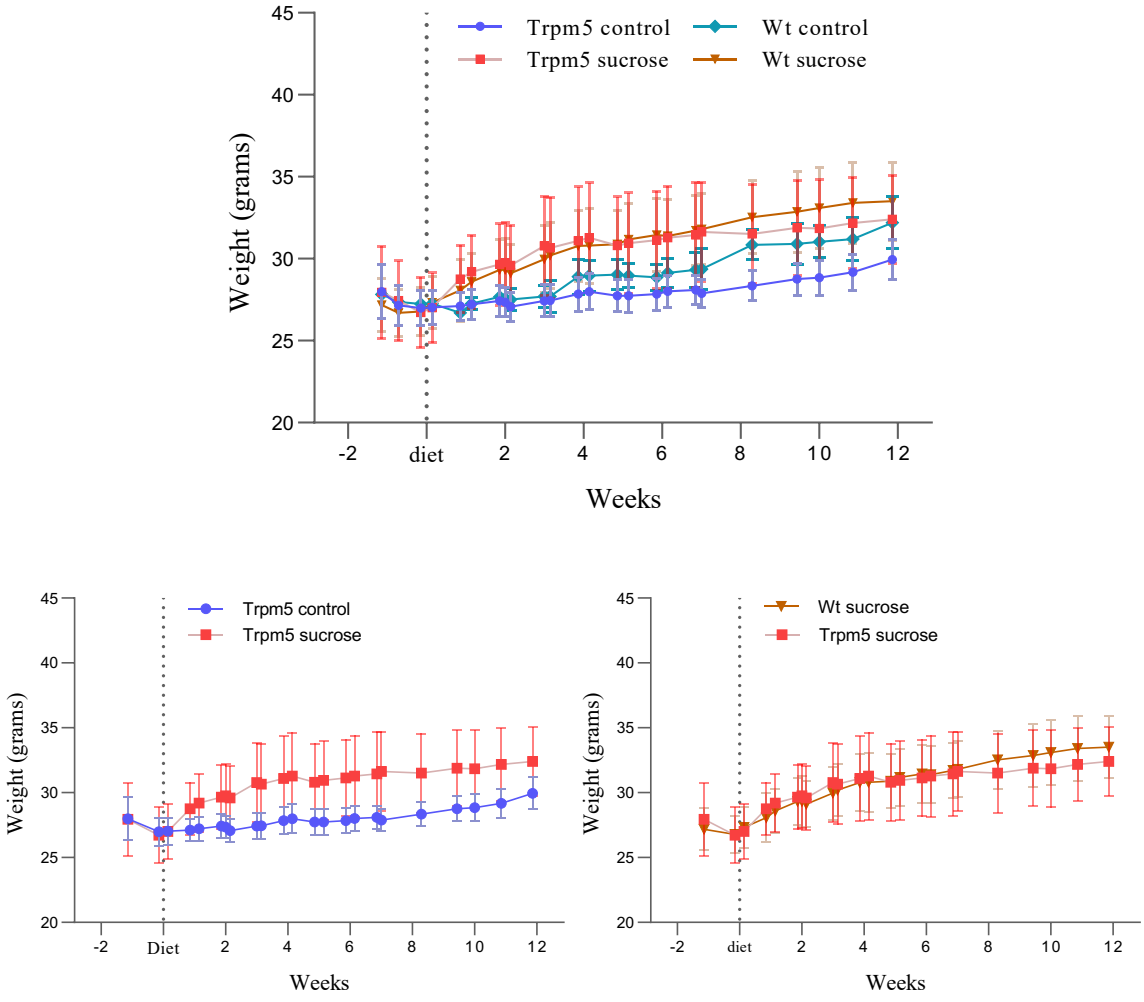


Figure 4.7. Results of weight in Trpm5 knock out mice (Trpm5) and control littermates (WT) mice relative to the mean of one week prior to DIO exposure and during the 35% sucrose solution DIO protocol along 12 weeks. Top. Weight(g) of Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4) vs. Trpm5 control (blue circles, N=5) and WT submitted to 35% sucrose solutions (orange triangles, N=7) vs. WT control (turquoise rhombus, N=4) **Right.** Weight(g) of Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4) vs. Trpm5 control (blue circles, N=5). **Left.** Weight(g) of WT submitted to 35% sucrose solutions (orange triangles, N=7) vs. Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4). Two-way ANOVA was performed.

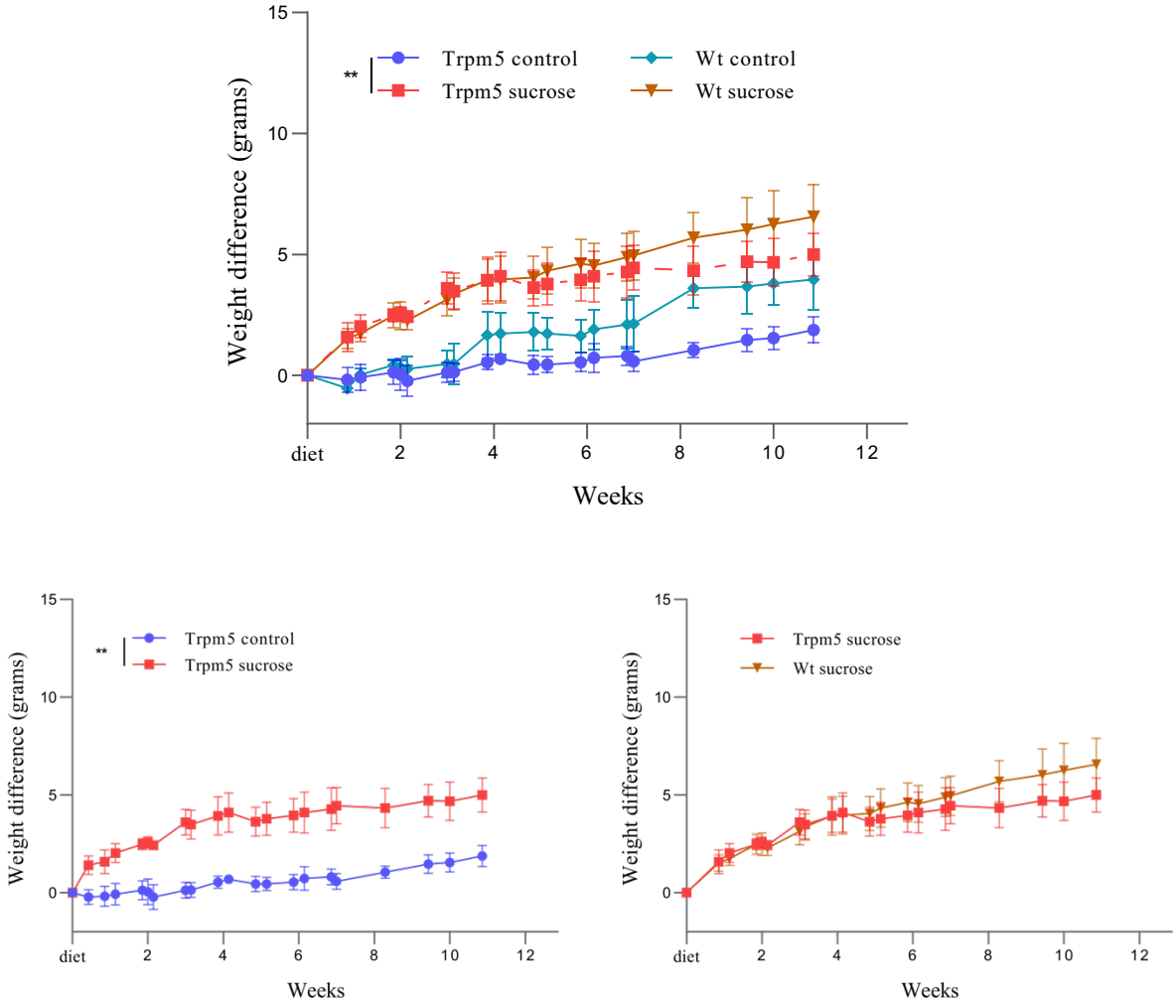


Figure 3.8. Results of weight difference in Trpm5 knock-out mice (Trpm5) and control littermates (WT) mice relative to the mean of one week prior to DIO exposure and during the 35% sucrose solution DIO protocol along 12 weeks. **Top.** Weight difference(g) of Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4) vs. Trpm5 control (blue circles, N=5) and WT submitted to 35% sucrose solutions (orange triangles, N=7) vs. WT control (turquoise rhombus, N=4) **Right.** Weight difference(g) of Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4) vs. Trpm5 control (blue circles, N=5). **Left.** Weight difference(g) of WT submitted to 35% sucrose solutions (orange triangles, N=7) vs. Trpm5 mice submitted to 35% sucrose solutions (red squares, N=4). Two-way ANOVA was performed. **p=0.0027.

In this group, food and water consumption was also analyzed. Trpm5 mice did not differ significantly from the WT mice. Both groups when exposed to 35% sucrose decreased significantly food consumption and increased significantly sucrose consumption (figure 3.9.). This latter is probably responsible for the increase in body weight observed in both strains.

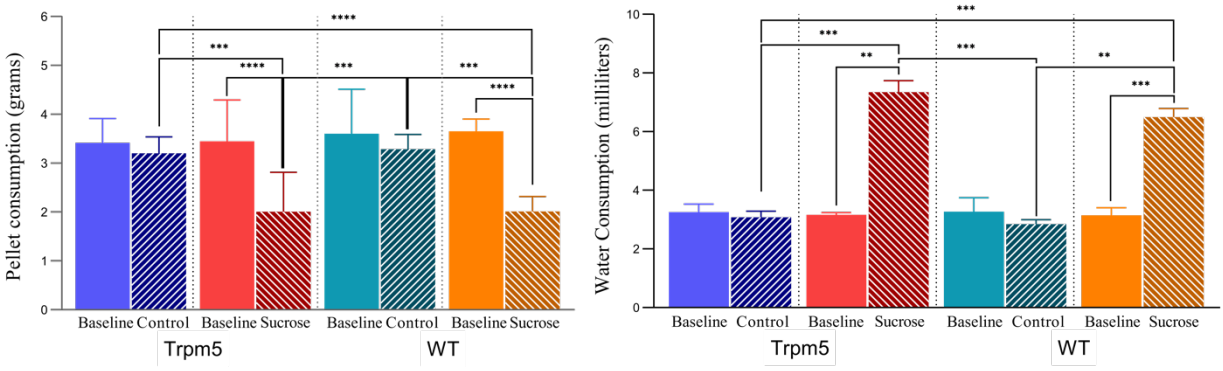


Figure 3.9. Results of pellet and water consumption of Trpm5 knock-out (Trpm5) and wild type (WT) mice during the 35% sucrose solution DIO protocol along 12 weeks. Left) pellet consumption of Trpm5 mice submitted to 35% sucrose solutions (red) vs. control (blue) and WT mice submitted to 35% sucrose solutions (orange) vs. WT control (turquoise). The solid bars correspond to the consumption in the baseline (before starting the DIO), the pattern bars correspond to the data during the DIO protocol. Pellet consumption: Trpm5 control vs. tprm5 sucrose ***p=0.0001; Trpm5 control vs. WT sucrose ****p=<0.0001; Trpm5 baseline vs. Trpm5 sucrose ****p<0.0001; Trpm5 sucrose vs. WT control ***p=0.0003; WT control vs. WT sucrose ***p=0.0001; WT baseline vs.WT sucrose ****p<0.0001. Water consumption: Trpm5 control vs. tprm5 sucrose ***p=0.0002; Trpm5 control vs. WT sucrose ***p=0.0005; Trpm5 baseline vs. Trpm5 sucrose **p=0.0014; Trpm5 sucrose vs. WT control ***p=0.0004; WT control vs. WT sucrose ***p=0.0008; WT baseline vs.WT sucrose **p=0.0015.

4. Discussion

Firstly, our results reveal that neurons in the VTA increase their activity after post-ingestive stimulation. This response depends on the nutrient infused in the stomach being specific for carbohydrates but not for isocaloric solutions such as fat or protein.

Albeit previous evidences propose that nutrient detection can be important to feeding behaviour using behavioural food preference tests where flavours or locations paired with nutritive solutions are preferred rather than those paired with non-nutritive solutions (de Araujo et al., 2008; Touzani et al., 2008), there is no evidence that post-ingestive VTA signals increase after post-ingestive feedback. The measure of VTA dopaminergic neurons in freely moving mice is an approach that is only possible due to the development of this new technique that allows for the visualization of neurons with spatial and temporal resolution in deep brain nuclei (Resendez et al., 2016). Until now the measures available could only infer indirect measurements of dopaminergic release in the striatum. Araujo et al was one of the authors that explored the rewarding effects of post-ingestive signals and striatal dopamine release, but their results throughout the publications show very inconsistent results in relation to the striatal region that is activated by these mechanisms (de Araujo et al., 2008; De Araujo et al., 2012, 2013). Also, the dopamine modulation with different nutrients was never explored. We have shown for the first time that post-ingestive mechanisms activate directly VTA dopamine neurons and this activation occurs only in the presence of intragastric sucrose infusion and not of isocaloric solutions of corn oil or glutamate. These results are in accordance with several other studies that have shown that mice develop clear behavioural preferences for carbohydrates in flavour-nutrient paradigms. However behavioural flavour-nutrient paradigms have also shown that mice develop preferences for fat indicating that fat could also have post-absorptive effects that lead to activation of rewarding areas within the brain (Sclafani, 2004; de Araújo et al., 2019). We did not observe clearly this effect when corn oil was infused which does not exclude that other dopamine nuclei respond to post-ingestive fat content. In fact, Araujo et al have infused an intralipid solution in animals and showed that substancia nigra compacta (the other nuclei that releases dopamine) and dorsal striatum were the main areas activated by fat (De Araujo et al., 2012; de Araújo et al., 2019). Our results, in accordance with the ones previously published, suggest that dopamine in the VTA probably encodes specifically sucrose post-ingestive mechanisms that are highly rewarding and reinforcing. This result is also corroborated by the fact that ingestion of pure fat is not a pleasant experience indicating that the brain is definitely much more prone to sense and attribute rewarding and reinforcing properties to sweet reinforcers rather fat or protein reinforcers. The cellular and molecular machinery by which post-ingestive sucrose leads to increased VTA dopaminergic neuron activity is not completely known. Unpublish data from Oliveira-Maia lab showed that post-ingestive carbohydrate dependent VTA activity is partly sensed through the vagus nerve. Recently Kaelberer et al have shown that sensing of glucose in the gut also occurred through the vagus nerve, a mechanism involving neuropod cells (Kaelberer and Bohórquez, 2018; Steinbusch et al., 2015; Tellez et al., 2013). Despite these, the physiological relationship between excess carbohydrate intake and brain VTA dopamine remains elusive. With an attempt to validate a causal correlation between food stimuli and weight gain we developed a second aim which intended to study the importance of post-ingestive stimulation during a protocol were obesity was induced by diet manipulation. Due to time constrains we were not able to develop entirely this aim but we were able to first understand the most effective diets that robustly induced weight gain. Based on our results animals do have an outstanding capacity to control their metabolic state. Nevertheless, and as previously described (Surwit et al., 1995) high fat diet are

one the most effective diets in inducing weight gain, especially if the content of fat is very high (above 50%). Since our final objective was to couple with weight gain VTA dopaminergic activity, it was mandatory to have a diet that would induce rapid weight gain. On the contrary animals exposed to a High Carbohydrate diet were not able to increase weight gain, probably due to low palatability. Manipulating diets in relation to the carbohydrate content is risky since several evidence indicate that animals would not significantly increase body weight. Most of these 70% carbohydrate diets are assigned for development of several metabolic alterations associated to type2 diabetes without the development of obesity.

Alteration of pellets composition is an all-area of study and several diet companies still try to understand why an animal when exposed to a high fat or high carbohydrate diet decrease food consumption. This was also a result that we observed. The palatability of these diets seems to be very different and unpleasant that ultimately make animals decrease their food consumption. Independent of food consumption HF diet was able to induce significant increases of weight gain probably due to their 60% of fat. The ratio of calories per g was higher is this diet (5.1kcal/g) when compared to the high sucrose diet (4.0Kcal/g) indicating that the caloric content ingested in the animals exposed to HFD was higher and probably sufficient to induce weight gain. In addition, the control group also showed a decrease in food consumption, although we cannot explain this unexpected result, it might be due to the fact that in this group there are differences in the age and laboratory origin of the animals (Champalimaud Foundation vivarium and The Jackson Laboratory®). A re-test with a more homogenous sample of animals could be done to check the significant differences of the control group.
It is also important to refer that controversy exists regarding macronutrient-preference in obesity. While some human studies report increased preference and consumption of carbohydrates in obese compared to non-obese individuals, other studies suggest a major role of dietary fat (Heraief et al., 1985; Lieberman et al., 1986; Wurtman and Wurtman, 1984). Consistent with human findings, animal studies also demonstrate increased fat preference in obesity, as evidenced by a macronutrient choice procedure (Okada et al., 1992). However, most high palatable and density food share these two macronutrients, thus the development of obesity should be caused by synergetic effects of both nutrients acting together. For this reason and because it was important to have a liquid pure diet to, if possible, infuse directly into the stomach we also explored the effect of inducing obesity with a 35% sucrose solution (substituted by the normal water). Studies reported in different mice strains, including the B6 strains, a higher consumption of sweet solutions when compared to water, showing preference for sweet solutions, accompanied by a weight gain (Lewis et al., 2005; Glendinning et al., 2012). Our results are in accordance with the previous studies, showing a significant increase in weight gain that was accompanied by a significant increase in 35% sucrose solution consumption. This increased consumption and preference probably explained why these mice eat less regular pellets.
In summary our results regarding the diets manipulation reveal that both 35% sucrose solutions and High-Fat diets are capable of inducing obesity, however, the most efficient in time-related effects, was the High-Fat, showing effects as early as the third day of diet. This can be explained due to high caloric content of these diets (60% fat).

After exposing animals to different animals and since we were able to see that liquid pure sucrose diet was able to induce weight, we decided to check what would be the response of a mutant mice where orosensorial sweet feedback was impaired. Trpm5 KO mice are not able to differentiate between a water and a sucrose solution, as previously published (Zhang et al., 2003). However, after a behavioural paradigm where sucrose was conditioned to one side of a cage a clear preference for this sucrose side when compared to a sucralose place conditioning protocol was observed (Oliveira-Maia et al., 2011). Oliveira Maia et al concluded that Trpm5 mice were able to develop a preference for the sucrose sipper

based on post-ingestive mechanisms indicating that these mechanisms can modulate feeding behaviour independent of orosensorial feedback. Based on this previous result we decided to induce obesity in these mutant mice in order to understand the impact of post-ingestive feedback in DIO. Our results show that Trpm5 mice do not show any difference in weight gain or consumption patterns when compared to the control littermates mice when exposed to a 35% sucrose diet indicating that post-ingestive feedback is sufficient to sustain feeding decisions leading to weight gain.

5. Final Considerations

This work provided new insights regarding the post-ingestive signaling function in the VTA dopamine neurons. It was possible to see that carbohydrate post-ingestive stimulation increased VTA dopaminergic neurons, positioning these mechanisms as important modulators of feeding behaviors. It was also concluded that VTA dopaminergic responses dependent on post-ingestive mechanism is specific for carbohydrates. Isocaloric solutions such as fat and protein did not induce the same alteration as sucrose infusion. It is important to highlight that the results presented are relative to one mouse and to 2 sessions for each reinforcer. Thus, in future it is extremely important to perform these experiments in a large number of animals.

Unfortunately, in this work it was not possible to conclude the aim 2, although we were able to optimize the methods and found the most effective DIO to conclude this aim. Therefore, future studies will address the questions related to aim 2 in the work developed, and further explore the role of dopamine signaling in the VTA neurons during the development of obesity, specifically how post-ingestive mechanism modulate weight gain and dopamine responses, using a DIO protocol. It would be also interesting to investigate this question with other sample sugars reinforcers and also in a female group, but in order to do this, it should also be done an DIO optimization for the female group.

It will be also important and interesting to explore the how the post-ingestive signals are sent from the gut to the brain. Although there is no certainty yet, a few works suggest that the vagus nerve is a good candidate, due to the fact that the portal vein in the liver is the first site of glucose accumulation after food absorption, and that this vein connects to vagus nerve of the hepatic branch. Thus, further experiments in order to the determine if it is the vagus nerve responsible to the gut post-ingestive information to the brain will be conducted in a near future.

In conclusion, the results obtained associated with future approaches can clarify the role of neural aspects influencing weight gain. It can clearly elucidate important alterations of pathways and infer causality in impaired feeding patterns such as obesity that ultimately can generate useful powerful tools for possibly find better innovative and less invasive treatments for obesity.

6. References

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